PRODUCTION OF FERULIC ACID FROM BANANA STEM WASTE BY USING CO-CULTURE

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ABSTRACT

Ferulic acid (FA) or known as (E)-3-(4-hydroxy-3-methoxy-phenyl) prop-2-enoic acid is a type of caffeic acid derivative. It is widely found in vegetables, fruits and some beverages such as beer and coffee. The abundance of these aromatic compounds in plant materials combine with known microbial transformation made them attractive for biotransformation research. Therefore production of FA by co-culture was a main focus in this study. This research study consists of two objectives. First objective was to perform growth profile for co-culture and produce FA from banana stem waste, thus test for the best producer. Second objective was to perform kinetic study of FA production by using co-culture. Possibility of using cheap materials in this research study had been proven as the co-culture was isolated from soil and banana stem waste (BSW) as the substrate. The method for this research study was divided into six steps. Firstly, substrate was prepared by collecting BSW at Gambang, Pahang, Malaysia. Then, the inoculum for pure culture was prepared to perform the fermentation process. After 24 hours of fermentation, they were ready for the analysis by using HPLC. Based on the analysis, six best producers from pure culture were determined as the best producers which are bacteria *Bacillus thuringiensis* strain NRBC101235 (A), *Brevibacillus formosus* (B), *Bacillus pumilus* SAFR-032 (C), *Bacillus cereus* strain JCM 2152 (D), *Lysinibacillus fusiformis* strain NRBC15717 (E) and *Bacillus cereus* strain ATCC14579 (F). Then, growth profiles for pure culture and co-culture were carried out by using indirect method. From the growth profile result, two sets of inoculum for co-culture were prepared and injected into substrate at their stationary phase. The co-culture was from combination of bacteria ABEF and BCDEF. Then, fermentation process began. From the analysis, the set of co-culture BCDEF was identified as the best producer. They were able to produce FA from the range of 2.0 to 3.0 mg/g. According to the previous research, the concentration of FA should be from the range of 1 to 3 mg/g. Kinetic study for co-culture BCDEF was carried out. The hyperbolic equation proposed by Monod which modified by Lawrence and McCarty was used to describe microbial growth and biodegradation process. In order to solve the equation, the values of kinetic parameters which are Ks, q_{max} and Y were calculated by using Runge-Kutta Fourth method. The values obtained were 0.9 g/L, 0.1669 mmol/cell.h and 0.8077 g/g respectively with R-squared value for substrate and biomass were 0.8538 and 0.8833 each. Hence, FA was successfully produced from banana stem waste by using co-culture.

ABSTRAK

Asid Ferulik (AF) adalah sejenis asid “caffeic derivative”. Ia boleh didapati secara meluas di dalam sayur-sayuran, buah-buahan dan minuman seperti bir dan kopi. Apabila tumbuhan dicampurkan dengan transformasi mikrob, ia menjadikan mereka sebagai bahan tarikan untuk penyelidikan biotransformasi. Oleh itu pengeluaran AF dengan menggunakan ‘co-culture’ merupakan tumpuan utama di dalam kajian ini. Kajian penyelidikan ini terdiri daripada dua objektif. Objektif pertama adalah untuk melaksanakan profil pertumbuhan ‘co-culture’ dan menghasilkan AF daripada batang pisang, dan mengenal pasti pengeluar yang terbaik. Objektif kedua adalah untuk melaksanakan kajian kinetik pengeluaran AF dengan menggunakan ‘co-culture’. Kegunaan bahan kos rendah dalam kajian penyelidikan ini telah terbukti dengan menggunakan ‘co-culture’ yang telah diambil daripada tanah dan batang pisang sebagai substrat. Kaedah di dalam kajian penyelidikan ini telah dibahagikan kepada enam langkah. Pertama, substrat telah disediakan dengan mengumpulkan batang pisang terbuang. Kemudian, inokulum bagi ‘pure culture’ telah disediakan untuk melakukan proses penapaian. Berdasarkan analisis, enam pengeluar terbaik daripada ‘pure culture’ telah ditentukan sebagai pengeluar terbaik iaitu bakteria *Bacillus thuringiensis* NRBC101235 (A), *Brevibacillus formosus* (B), *Bacillus pumilus* SAFR 032 (C), *Bacillus cereus* JCM 2152 (D), *Lysinibacillus fusiformis* (E), dan *Bacillus cereus* ATCC 14579 (F). Kemudian, profil pertumbuhan ‘pure culture’ dan ‘co-culture’ telah dijalankan dengan menggunakan kaedah tidak langsung. Dari hasil profil pertumbuhan, dua set inokulum untuk ‘co-culture’ telah disediakan dan disuntik ke dalam substrat. Kumpulan ‘co-culture’ adalah daripada gabungan bakteria ABEF dan BCDEF. Daripada analisis, ‘co-culture’ BCDEF telah dikenal pasti sebagai pengeluar yang terbaik. Mereka dapat menghasilkan AF dari lingkungan 2.0 hingga 3.0 mg/g. Menurut kajian sebelum ini, kepekatan AF harus dari lingkungan 1 hingga 3 mg/g. Kajian kinetik untuk ‘co-culture’ BCDEF telah dijalankan. Persamaan hiperbolik dicadangkan oleh Monod yang diubahsuai oleh Lawrence dan McCarty telah digunakan untuk menggambarkan pertumbuhan mikrob dan proses biodegradasi. Dalam usaha untuk menyelesaikan persamaan, nilai parameter kinetik iaitu Ks, qmax dan Y telah dikira dengan menggunakan kaedah Runge-Kutta Keempat. Nilai-nilai yang diperolehi ialah 0.9 g/L, 0.1669mmol/cell.h dan 0.8077 g/g, masing-masing dengan nilai R^2 untuk substrat dan biomass adalah 0.8538 dan 0.8833. Oleh itu, AF telah berjaya dihasilkan daripada batang pisang dengan menggunakan ‘co-culture’.

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FA	Ferulic Acid
BSW	Banana Stem Waste
HPLC	High Performance Liquid Chromatography
GDP	Gross Domestic Product

1 INTRODUCTION

1.1 Motivation and statement of problem

Agricultural is one of the sectors which contribute to Malaysia's Gross Domestic Product (GDP) (D. Bilanović et. al., 2011) . Nearly twenty four percents of Malaysia's land area composed of land which dedicated to agriculture alone. Malaysian farmers produced high quality fruits and vegetables for domestic market consumption such as durian, coconuts, bananas, pineapples and paddy. Consequence to this activity, many agricultural residues left abundant. It may cause environmental pollution. By exploiting agricultural residue, it will solve the problem facing by most of the farmers. Banana plant is the largest herbaceous flowering plant (Kumar & Kumar, 2011). In fact, banana stem waste (BSW) is one of the agricultural wastes that can be found easily in Malaysia. Moreover, it is available in large quantity after harvesting. Banana stem cell wall contain lignocelluloses materials (Cruz et. al., 2001). There are three main components of lignocelluloses which are cellulose, hemicellulose, and lignin (Ibrahim et al., 2010). Cellulose and hemicellulose can be hydrolysed with chemicals and/or enzymes to monomeric sugars, which can subsequently be converted biologically to any bio products (Hasyierah et al., 2011). Hence, it can be used as an alternative source of phenolic acid production.

Ferulic acid (FA) [(E)-3-(4-hydroxy-3-methoxy-phenyl) prop-2-enoic acid] is belongs to the family of phenolic acids (Mancuso & Santangelo, 2014). FA is a chemical compound that plays role in various biochemical processes. It grabs all the attention as a chemical with many potential applications. FA had been used for several applications especially in the food, health, cosmetic and pharmaceutical industries (Ou and Kwok, 2004). Therefore, recovery of FA from BSW becomes very important to fulfil the market demand in large scale of production. **Error! Reference source not found.**.1 below shows the chemical structure of ferulic acid.

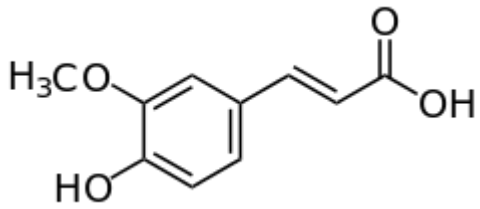

Figure 1.1: Chemical structure of ferulic acid

Fermentative production is highly recommended by using cheap by-products and waste substrates (S. Dominik and J. Anna, 2011). The main problem in producing FA is to find raw materials which are environmental friendly and cheap. Most of the materials used for FA production are sugar based feedstock. There were several researches on FA production from waste such as wheat bran, paddy straw (Hasyierah et al., 2011) and wheat straw. According to Papagianni et al (2001), chemical purity is mainly depends on the essential part in the fermentation medium especially when cheap materials are being used. So, contrast to the statement, production of FA from BSW can be produce in large scale due to low cost raw material and easy to find in Malaysia. This second largest tropical fruit cultivation has led to generate several tons of underuse by-product and waste. So, by exploiting this residue, it will reduce ecology damage due to improper agricultural management practice. This is an advantage to take the BSW as the raw material and study their FA production.

Apart from that, many researchers had been reported in releasing FA by using only a single pure culture. Sometimes the process needs more than one culture to release FA efficiently. Co-culture fermentations may result in increased yield, improved control of product qualities and the possibility of utilizing cheaper substrates. Co-cultivation of different microorganisms may also help to identify and develop new biotechnological substances. As Bertrand et al (2014) had mentioned in their findings, the co-production of enzyme by microorganisms is very important to increase the production of ferulic acid. Hence by using soil co-culture, FA production from BSW can be produce in maximal yield. Co-culture provides better biomaterial to be used under physiological condition. Hence, in this study, the best condition to produce FA was proved by using co-culture which is facultative anaerobic bacteria and BSW as the substrate.

1.2 Objectives

The following are the objectives of this research:

- To perform growth profile for co-culture and produce FA from banana stem waste thus test for the best producer.
- To perform kinetic study of FA production by using co-culture.

1.3 Scope of this research

In this research study, scopes function as a guideline to achieve the objectives. As FA is most abundant in plant, BSW was used as the substrate. Firstly, BSW was collected at Gambang, Pahang, Malaysia. The co-culture was isolated from the soil where soil was collected from the banana farm. In order to keep the BSW moist, it was stored in the 4^{o}C refrigerator. To complete the first objective, 21 sets of pure cultures were grown on the agar plate. Then, BSW and nutrient broths were prepared. Nutrient broths were prepared for the inoculum process. After 24 hours streaking and 24 hours inoculum process, pure cultures were ready for the fermentation process. Samples containing BSW and pure cultures were incubated in an incubator shaker for 24 hours at 150rpm and 35^{o}C. On the next day, the production of FA was analyzed by using High Performance Liquid Chromatography (HPLC). Based on the data analyzed, the best producers for FA production were determined. There were six pure cultures determined. From them, two sets of co-cultures were prepared and analyzed again for the best co-culture producer. Before fermentation process for the co-culture started, growth profiles for each six types of pure cultures and two sets of co-cultures were studied. Method using was indirect method. This method using spectrophotometric measurements of the developing turbidity at the same 1 hour interval with total of 24 hours as an index of increasing cellular mass. Therefore, the optical densities (OD) of the co-culture were recorded. Identification of the best co-culture was done based on their contribution to produce FA by using BSW. Therefore, first objective was completed.

The best pure cultures were going through identification processed by using molecular method. This identification required PCR and cycle sequencing to isolate genomic DNA from bacteria. This protocol involved breaking the cells opened with a series of freeze/thaw cycles. PCR reaction was set up to amplify a region of the 16S rRNA gene. The cleaned PCR product was used as the template for a sequencing reaction was run in a thermocycler (PCR machine). The electropheragrams was viewed from the sequencing reaction and the sequence was used in a BLAST search limited to a bacterial data base. Unknown bacteria were identified by examining the top-scoring sequences from the BLAST search results. For the second objective, kinetic study of FA production from one set of co-culture which was the best producer was studied. Modified monod equation method was used in this study. BSW and biomass concentration data were collected by interval of 3 hour for total of 24 hours. Based on the data, Runge Kutta-Fourth method was used to calculate the kinetic constants which were Ks, q_{max} and Y.

1.4 Main contribution of this work

From this research study, it can enhance the knowledge about production of FA by using co-culture. The usage of co-culture is one of the best methods in the fermentation as it was able to release multi-enzyme in the process. Many researchers had been reported in FA released using only a single pure culture. However this process needs more than one pure culture to release FA in higher yield. Therefore the co-production of enzyme by microorganisms is very important to increase the production of ferulic acid (Bertrand et al., 2014). Nevertheless, co-cultivation of different microorganisms may also help to identify and develop new biotechnological substances. Besides that, degradation of FA from BSW was one of the initiatives in using cheap materials and waste as the substrate. As mentioned by Zhao S. et al (2013), even though corn bran has highest content of ferulic acid, corn bran has low value and often used for animal feed alone or in combination with corn germ cake or meal. Hence by using soil co-culture, FA production from BSW can be produce in maximal yield. In fact, the study helps to optimize the production of FA by scaling up the process. Considering the exponential increase in the number of papers on this topic that have been published last several years, the use of this method will expand rapidly and yield important and fascinating discoveries.

1.5 Organisation of this thesis

Chapter 2 provides a description of the FA, its characteristic and application. In addition, the advantages of using BSW as the substrates and soil microorganism were explained. The chapter also provides a brief discussion on growth profile of microorganism and kinetic study of FA production. Apart from that, additional information on composition of natural cellulosic feedstock also explained below. There are three composition of natural cellulosic which are lignin, cellulose and hemicellulose. A summary of the previous experimental work on FA fermentation was also presented.

Chapter 3 gives a review of the chemicals, raw materials and the methods for the fermentation process. This chapter presents the experimental setup in completing the laboratory works. It includes in performing growth profile of the microbe, fermentation process and analysis of FA by using HPLC. Lastly, kinetic study for FA production was carried out. In this microbial process, bacteria were isolated from soil and BSW as a substrate. Preliminary studies were conducted in determining the bacteria that was chosen to further with the research of FA production from co-culture. Selection of co-culture was based on the performance of the pure culture in the release of FA from BSW.

Chapter 4 is devoted on the fermentation process to determine the best producer of FA production. In this chapter, result and discussion for the experimental study were presented. There were results for fermentation process by using pure culture and co-culture and growth profile for six pure culture and two sets of co-culture. Apart from that, kinetic study result for co-culture also discussed here.

Chapter 5 draws together a summary of the thesis and recommendation which might be derived in this work.

2 LITERATURE REVIEW

2.1 Overview

This study presents a microbial fermentation study of FA production by using co-culture. There were several researchers had done their research in the production of FA. However, there are plenty of studies in the fermentation of FA by using co-culture. Most of the research studies were on the production of FA by using pure culture. Previous work on the production of FA was explained below. In addition, detail explanations about FA, soil microorganism, banana stem waste (BSW), composition of natural cellulosic feedstock, growth profile of bacteria and kinetic study by using Monod equation were explained below.

2.2 Introduction

Phenolic acids have been studied extensively due to their anti-oxidative, anti-inflammatory, and other health related properties. It had been demonstrated both in vitro and in vivo (Sarangi and Sahoo, 2009). FA was bound to the hemicellulose of plant cell walls which is ubiquitous hydroxycinnamic acid. Agricultural lignocellulosic residues are the major potential source of low-cost raw material for commercial FA production. In order to achieve it, many researchers did their study on FA production by using agricultural waste as the raw material. However, plenty of them were using co-culture to degrade the waste. Thus, this research mainly focuses on the production of FA by using banana stem waste and co-culture. There are promising studies on FA fermentation with co-cultures bacteria. The main challenge with co-culture fermentation is finding and providing the optimal environmental conditions for two different bacteria, simultaneously.

2.3 Previous work on Ferulic Acid

According to Karagöz and Özkan (2014), agricultural lignocellulosic residues are the major potential source of low-cost raw material to commercialize FA production. Therefore, most of the researchers were using agricultural material in their studies. FA is commonly found in grains such as corn, wheat, barley, rye, oats and rice (Perez et. al., 2005). Besides, it also contains in fruits, vegetables, and in a wide variety of plant tissues. In barley, rye and wheat, the levels of FA decrease with age. This is correlated with an increase in the biodegradability of the plant tissue. In corn, the concentration of FA in hulls is 2.0 to 4.0% of the dry weight. Typically, levels of FA are higher in the hulls or bran of grains.

Based on Sarangi and Sahoo (2009) research, commercial natural FA was obtained from wheat bran. Wheat bran is an important by-production of the flour industry. The bran contains some starch, protein and hemicelluloses. It also contains many phenolic acids, such as FA and vanillic acid. Besides that, FA can be produced by using rice bran. However, it is expensive due to the nutrition and γ-oryzanol it contained. Therefore, some alternative FA containing lignocellulosic materials have been investigated. One of the potential lignocellulosic materials is paddy straw (Hasyierah et al., 2011) as it is abundantly available and is currently under-utilized. For example, in Malaysia, the production of rice is reported to be 2.4 million tons/year. It was resulting into a huge production of rice-straw. Since this paddy straw is regarded as a waste, Hasyierah et al. (2011) had made an initiative to extract FA. Ferulic acid is found varying from 5 g/g in wheat bran and corn kernel, 9 g/g in sugar beet pulp, and 15−28 g/g of rice bran oil (Zhao et al., 2013). Although wheat bran is rich in FA, it is expensive due to the nutrition and γ-oryzanol it contained.

There are also researches of FA production by different microorganism. For example, production of FA from clove oil by *Pseudomonas fluorescens* E118. *Pseudomonas fluorescens* E118 contains abundant eugenol-degrading microorganisms. It is a clove-oil-tolerant strain, accumulated 6.1g/L FA under optimized culture conditions with the intermittent addition of eugenol (Musatto et. al., 2007). When the bacterium was applied to FA production, 5.8g/L FA was produced with the intermittent addition of clove oil. Since clove oil is much cheaper than eugenol, FA production from clove oil by the bacterium was promising for the industrial production of FA (Furukawa et al., 2003).

There was also release of FA from agroindustrial by-products by the cell wall-degrading enzymes produced by *Aspergillus niger* I-1472 (Bonnin et al., 2002). *Aspergillus niger* I-1472 was grown on sugar beet pulp to produce cell wall polysaccharide-degrading enzymes, including feruloyl esterases. These enzymes were tested to release FA from sugar beet pulp (Ferreira et al., 2007), maize bran, or autoclaved maize bran. They were efficient as the commercial mixture to release FA from sugar beet pulp (Ferreira et al., 2007).

As a result, they were much more efficient to release FA from maize bran after autoclaving pretreatment, as 95% of FA ester was solubilized. Scientific databases provide only limited knowledge on ferulic acid esterases (FAEs) produced by bacteria, especially by FA bacteria (Wang et al. 2004). Donaghy et al. (1998) screened 80 Bacillus-type strains and 50 gram positive bacteria (*Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus* and *Propionibacterium*) in agar-plate assay. The highest FAE activities were seen in *Lactobacillus fermentum* and *Bacillus subtilis* strains (McMurrough et. al., 1996)

2.4 Ferulic Acid

Ferulic acid is a derivative of cinnamic acid with the molecular formula of $C_{10}H_{10}O_4$. In 1886, Hlasiwetz Barth isolated 3-methoxy-4-hydroxycinnamic acid (P. Laure, 2007) from the genus *Ferula foetida* for the structure determination (Go, M. and Cantero, 2003). FA together with dihydroferulic acid is a component of lignocelluloses which conferring cell wall rigidity by cross linking lignin and polysaccharides (Idrees et al., 2013). Biosynthesis of FA and other cinnamic acids is from the amino acids phenylalanine or tyrosine. It is commonly found in seeds of plant such as rice, wheat and oats. FA in plants usually exists as the *trans*-isomer, although *cis*- *trans*-isomerization across the double bond has been reported (Ferreira et al., 2007).

The abundance of these aromatic compounds in plant materials and in agricultural by-products combined with the vast array of known microbial transformations of these compounds make them attractive for biotransformation research (Gerard, 1997). FA was first synthesized in 1925 by the Knoevenagel condensation reaction. It was by condensation of malonic acid with vanillin in the presence of quinolone or by Perkin condensation of vanillin with acetic anhydride. Synthetic FA has been characterized by C-NMR and X-ray crystallography. FA occurs as colourless orthorhombic needles when crystallized from hot water. It has a molecular weight of 194 and a melting point of 174^{o}C. FA is soluble in alcohol, ethyl acetate and hot water. However, it is moderately soluble in ether and sparingly soluble in benzene and petroleum ether (Gerard, 1997).

Isolation of FA from plant materials has been achieved by hydrolysis of FA esters with 10% H_2SO_4 followed by extraction with ethyl acetate. Separation and analysis of FA and other plant phenolic compounds has been accomplished with thin-layer chromatography (TLC), high performance liquid chromatography (HPLC) and standard column chromatography methods (Gerard, 1997). In nature, FA is found predominately esterified to a wide variety of compounds such as saccharides, glycoproteins, lignin, hemicellulose, quinic, carboxylic acids, fatty acids, betacyanins and sterols (Zhao et al., 2013).

The occurrence of FA in dietary food products and traditional medicinal preparations had prompted investigations into the pharmacology and toxicology of FA. For example, it has protective agents against UV-radiation-induced skin damage, treatment for cardiovascular diseases and cerebral thrombosis (Xie et al., 2010). Moreover, it has also been associated with other physiological effects (Mancuso and Santangelo, 2014), including inhibition of uterine contractions, anti-inflammatory, hypocholestrolemic and inhibition of viral replication. Studies of the feruloyl sterols found in rice bran oil (Gerard, 1997) showed that these ferulic acid esters caused numerous CNS effects. It includes increasing of spontaneous motor activity, decreased time to death and body temperature. In addition, FA exhibited biochemical role in the inhibition of seed germination, inhibition of indole-acetic acid and enzyme, inhibition of decarboxylation activity and other protective effect on microorganisms and pets (Zenno et al., 2003).

There are vast numbers of studies documented on the bio-medical properties of FA such as antioxidant activity (Shahidi et. al., 1999), UV-absorbing capacity and its effect of lignin as precursor in plants metabolic pathway. It is also listed as 'antioxidant' in the 'food additives' list. It has been reported to maintain the colour tone of Greenpeace, prevent discoloration of Green Tea and oxidation of banana turning black colour. Thus, it reduces bacterial contamination.

The synthesis of FA was established when FA was used as a precursor in the manufacturing of vanillin and malonic acid. Vanillin is the world's most highly prized natural flavour. It is one of the most important aromatic flavour compounds used in foods, beverages, perfumes and pharmaceuticals (Sarangi and Sahoo, 2009). Currently, 12,000 tons (Gerard, 1997) of synthetic vanillin is produced annually for the flavouring industry. The conversion of FA to vanillin has been reported to occur by *Fusarium solani* and *Paecilomyces variotii* via oxidation of 4-vinylguaiacol. It is also has been isolated from *Bacillus subtilis* when incubated with FA (Mancuso and Santangelo, 2014).

Form the previous research, free FA, feruloyl glucose and feruloylputrescine are found in citrus juices such as orange juice. These compounds are contributed to the flavour of the juice. Thus, considering the increasing interest in 'natural' products, the production of flavours via biotechnological processes offers a viable alternative to natural and chemical sources (Sarangi and Sahoo, 2009).

Currently, two methods were developed to break the cross-link and release FA from plant cell wall materials. First, an enzymatic method using FAEs and the other is alkaline hydrolysis which release FA from polysaccharides which was often used to determine the content of FA in bran (Oosterveld et al. 2000). Ferulic acid esterase (FAE) or feruloyl esterase is a subclass of carboxylic acid esterase. It catalyses the hydrolysis of the ester bond between hydroxycinnamic acids and sugars on the plant cell. FAEs can be classified into four subclasses, type A-D, based on the activities toward a set of synthetic substract (Crepin et al. 2004).

The activity of ferulic acid esterase was first discovered in culture filtrates of *Streptomyces olivochromogenes* and *Schizophyllum commune* (Settachaimongkon et al., 2014). Studies show that the type of purified ferulic acid esterase from *Streptomyces olivochromogenes* and ferulic acid esterase (FAE-I) from *Aspergillus niger* alone could not release ferulic acid from wheat bran. However, purified ferulic acid esterase (FAE-II and FAE-III) from *Aspergillus niger* has been shown in isolation to release ferulic acid from wheat bran and barley spent grains. Each ferulic acid esterase also has its own specificity with regard to the release of specific cinnamic acids such as ferulic acid, ρ-coumaric acid, sinapic acid or caffeid acid.

2.5 Soil microorganism and co-culture

Microorganisms have a long track record as important sources of novel bioactive natural products, particularly in the field of drug discovery (R. Roberts, 1998). Microbes have been shown to biosynthesize a wide array of molecules. Recent advances in genome sequencing have revealed that such organisms have the potential to yield even more structurally diverse secondary metabolites.

In the last ten years, several methods have been developed to aid in the activation of these cryptic biosynthetic pathways. One of these approaches is microorganism co-culture. It involved the cultivation of two or more microorganisms in the same confined environment. Co-culture was defined as anaerobic or aerobic incubation of different specified microbial strains under aseptic conditions (J. Bader et al.,2009). Microorganism co-culture is inspired by the natural microbe communities that are omnipresent in nature. Within these communities, microbes interact through signalling or defence molecules. Microorganism co-culture can be achieved in either solid or liquid media and has recently been used increasingly extensively to study natural interactions and discover new bioactive metabolites.

Because of the complexity of microbial extracts, advanced analytical methods are key for the successful detection and identification of co-culture induced metabolites (Bertrand et al., 2014). According to J. Bader et al (2009), the advantageous utilization of co-cultures instead of single cultivations includes the production of bulk chemicals, enzymes, food additives, antimicrobial substances and microbial fuel cells. Co-cultivation of different microorganisms may also help to identify and develop new biotechnological substances. The relevance of co-culture fermentations and the potential of improving existing processes as well as the new production of new chemical compounds in industrial biotechnology can be pointed out. Some examples for the coexistence of different micro-organisms are the forest soils, compost piles, the aerobic and the anaerobic zones of water, spontaneous fermentations of sugar-containing saps and the human skin.

Co-cultures of different microorganisms may be also advantageous for the production of enzymes. One example is the production of laccases (García et al., 2011). These enzymes are able to hydrolyse the polymer lignin and may allow the utilization of this complex biopolymer for the production of fine chemicals. Further applications of laccases may be the decolorization of textile dyes or the production of biosensors. Transition elements such as manganese or phenolic compounds results in cost-intensive waste water treatment. Another industrially important enzyme is tannase (Ferreira et al., 2007). It is used in food, feed, pharmaceutical and textile industry. These biological approaches may be an emnvironmentally friendly and cost-saving alternative for the production of these enzymes.

The usage of soil microorganism was performed in this study. Soil contains variety of microorganisms including bacteria that can be found in any natural ecosystem. Microorganisms play an important role on nutritional chains that are an important part of the biological balance in the life in our planet. Without bacteria, soil would not be fertile and organic matter such as straw or leaves would accumulate within a short time (Kummerer, 2004). The use of co-cultures of a single strain of a microorganism is a method that has the advantages of being reproducible and amenable to further process development (Gerard, 1997). Furthermore, some fermentation process needs more than one enzyme to release FA efficiently. Therefore the co-production of enzyme by microorganisms is very important to increase the production of FA (Zenno and Iwasawa, 2003).

2.6 Microbial co-culture fermentation

Co-culture is an anaerobic or aerobic incubation of different specified microbial strains under aseptic conditions (Kummerer, 2004). Chemical substances produced each year such as fuels, fine chemicals and pharmaceuticals worth several billion Euros by biotechnological processes using renewable resources (Lemos et al., 2014). Because of the sterile cultivation enables an easy way of controlling microbial milieu, growth and product formation, most of the products in industrial biotechnology today are formed using processes involving a single microbial strain (J. Bader et al., 2010). On the other hand, there are many instances where the utilization of co-cultures appears to be advantageous over a single microorganism. It is because, the potential for synergistic utilization of the metabolic pathways of all involved strains in a co-culture situation. Most biotransformation in nature takes place by the combination of metabolic pathways from different microorganisms (Bertrand et al., 2014). Moreover, co-cultivation may result in increased yields, a reduction of process costs because of cheaper substrates (Kleerebezem and Van Loosdrecht, 2007) and control of product quality.

In some cases, production of substances normally not formed by pure cultures can be observed through the induction of appropriate genes in co-cultivation processes. Co-culture fermentation may have a great impact on the development of biofuels, bioenergy and bio-based products. Examples of the utilization of co-cultures in food industry are the production of cheese, yoghurt, sourdough, African fermented dairy products and Belgian beer such as Lambic. In co-cultures, degradation and metabolization of substrates occur by the combined metabolic activity of the known microbial strains under aseptic conditions (J. Bader et al., 2010). Modification of raw materials during food production by co-cultures results in improved texture, taste and flavor and in microbial stabilization. This protection may be caused by a decreased pH-value or by the formation of growth-inhibiting substances such as lactic acid, acetic acid and ethanol.

Apart from that, energy consumption and the use of environmentally hazardous substances can often be reduced by biotechnological production processes (Bertrand et al., 2014). Further advantages may be the production of pure enantiomers, reduced steps required in synthesis of products, and less stringent security needs resulting in reduced production costs (Gerard, 1997). The risk of accidents decreases as a result of lower process temperatures and normally low pressures in biotechnological processes. Moderate process conditions result in lower required charge in the field of process security and approval procedures. These biological approaches may be an environmentally friendly and cost-saving alternative for the production of these enzymes.

The controlled cultivation of co-cultures enables the synergistic utilization of the metabolic pathways of the participating microorganisms under industrial, reproducible and controlled condition (Papagianni et. al., 2001). The optimal values of process parameters are pH, temperature, oxygen demand and the acceptable ranges of substrate and product concentrations have to be known and considered to achieve the controlled fermentation, as in pure culture cultivation (Pinar and Melek, 2014). In co-culture fermentation processes, the complexity of possible interactions which are positive or negative has to be taken into account. All aspects, the process parameters, the produced and secreted substances and possibly the occurring biotransformation, may provide an opportunity to control growth and product formation during co-culture fermentation processes. Parameters have to be found enabling the utilization of the desired part of the metabolic pathway of every single strain in co-culture to achieve the development of a controlled co-culture fermentation process and to form the favored product (J. Bader et. al., 2010).

2.7 Banana Stem Waste (BSW)

Banana is the common name for herbaceous plants of the genus *Musa* (Alwi et al., 2013) and for the fruit they produce. Banana (*Musa spp.)* is an important world food crop. It is grown and consumed in more than 100 countries throughout the tropics and subtropics. In developing countries, they are four most important food crops after rice, wheat and maize. Some study shows that there are higher content of several phenolic compounds such as dopamine and cyanidin-related compounds, lignin, and FA (Dom et al., 2008) were observed. In Malaysia, the production of commercial varieties of banana has increased by 24–27% (Alwi et al., 2013) over the decades. It gives an amount of 27,453 hectares in 2009 with Johor, Pahang, and Sarawak as the largest banana-producing states.

Bananas come in a variety of sizes and colours when they are ripe. The colours are yellow, purple, and red. In popular culture and commerce, "banana" usually refers to soft and sweet dessert. According to Alwi et al (2013), many varieties of bananas are perennial. The banana plant is the largest herbaceous flowering plant. Plants are normally not very tall. Their main or upright stem is called pseudostem that grows 6 to 7.6 meters tall. They grow from a corm. Each pseudostem can produce a single bunch of banana. After fruiting, the pseudostem dies, but offshoots may develop from the base of the plant. Leaves are spirally arranged and may grow 2.7 meters long and 60 cm wide. They are easily torn by the wind, resulting in the familiar frond look.

Each pseudostem normally produces a single inflorescence or known as the banana heart. The inflorescence contains many bracts or called as petals in between rows of flowers. The female flowers which can develop into fruit appear in rows further up the stem from the rows of male flowers. The ovary is inferior, meaning that the tiny petals and other flower parts appear at the tip of the ovary. Banana fruit develop from the banana heart, in a large hanging cluster, made up of tiers with up to 20 fruit to a tier. The hanging cluster is known as a bunch, comprising 3–20 tiers, or commercially as a "banana stem", and can weigh from 30–50 kilograms. In common usage, bunch applies to part of a tier containing 3-10 adjacent fruits.

Major factor in the economic production of FA is the cost of raw material. By-products of agriculture industries are one of the alternatives substrate and renewable resources for FA fermentation. Depending on the availability of the substrate in the country, variety of glucose was used to produce FA such as lignocellulosic biomasses (Lithchfield, 1998). Lots of studies have been done by using various agriculture resources such as wheat straw (Karagoz and Ozkan, 2014), wheat bran (Xie et al., 2010), paddy straw (Hasyierah et al., 2011) and corn. Banana stem waste (BSW) is one of the new renewable sources that have been used as substrate. According to the Table 2.1, Alwi et al., (2013) stated that glucose content in the banana pseudo stem is 74.0% higher compared to the others. The statement also supported by Sinha et al., (2012), where banana stem-central core contain 1.20% of carbohydrates per 100 gram. Therefore, in this study, the using of BSW as the substrate was established. As banana stem has no use after harvesting the fruit, it is good to make them as the carbon source for the ferulic acid fermentation process rather than throw them away.

Banana stem waste is a lignocelluloses waste, which consists of lignin, cellulose and hemicellulose. It consists of 15.42% lignin, 53.45% cellulose and 28.56% hemicelluloses (Alwi et al., 2013). In general, according to Romero et al., (2011) banana stem has potential for providing products such as manure and feet, but this practice only processes a small fraction of the total waste production. Through the exploitation of this waste material, high value compound is very significant and promising high profits with low cost (Marcia et al., 2007).

In biological pre-treatment, microorganisms, mostly fungi, are used to digest lignin and hemicellulose in waste materials. White-rot fungi such as *Pleurotus ostreatus* and *Pycnoporus cinnabarinus* 115 are preferred for biological pretreatment because of its high efficiency in degrading or modifying the lignin content in lignocellulosic biomass. Besides, some modifications have been made to fungal cultivation to improve the digestion of lignin and avoid degradation of cellulose. However, microorganism was used in this study to degrade the lignin and hemicellulose (Bajpai and Stahl, 2010).